Preeclampsia

Fibrinogen-Like Protein 2-Associated Transcriptional and Histopathological Features of Immunological Preeclampsia

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Abstract—Preeclampsia is a multifactorial hypertensive disorder of pregnancy, with variable presentation in both maternal and fetal factors, such that no treatment or marker is currently universal to all cases. Here, we demonstrate that the prothrombinase and immunomodulatory secreted factor FGL-2 (fibrinogen-like protein 2) is differentially expressed across previously characterized gene expression clusters containing clinically relevant disease subtypes. *FGL2* is low in a cluster consistent with the traditional paradigm of the pathology of preeclampsia (canonical preeclampsia) and high in a cluster exhibiting evidence of immune activation (immunological preeclampsia). We show that it is part of an immunoregulatory gene module integral to the transcriptional profile and placental pathology specific to immunological preeclampsia. We determine that *FGL2* associates positively with chronic inflammation lesions of the placenta while associating negatively with maternal vascular malperfusion lesions. The transcriptional profiles of maternal vascular malperfusion lesions show downregulation of *FGL2* and upregulation of previously investigated preeclampsia biomarkers, such as FLT1 (Fms Related Receptor Tyrosine Kinase 1) and ENG (endoglin). Conversely, the profiles of chronic inflammation lesions show an interesting downregulation of these genes, but an upregulation of *FGL2* and of *FGL2*-correlated immunoregulatory genes, suggesting it is upregulated downstream of major inflammatory mediators such as TNF (tumor necrosis factor)-α and IFN (interferon)-γ, hallmarks of the immunological preeclampsia subtype. This work, overall, demonstrates that FGL-2 expression levels in the term placenta reflect the unique pathophysiology that leads to immunological preeclampsia, leading to its potential as a subtype-specific biomarker. (*Hypertension*. 2020;76:910-921. DOI: 10.1161/HYPERTENSIONAHA.120.14807.)

● Data Supplement

Key Words: chorionic villi ■ fibrin ■ histology ■ inflammation ■ placenta ■ preeclampsia

Preeclampsia is a hypertensive disorder affecting 3% to 8% of pregnancies, diagnosed in the mother following new-onset hypertension after 20 weeks of gestation, when accompanied by signs of end-organ damage.[1–3] The onset and severity of maternal symptoms and fetal health outcomes are highly variable across all preeclampsia cases.[1] To date, there remains no effective therapeutic intervention to prevent or cure preeclampsia, aside from delivery of the placenta.

Decades of research have led to a widely accepted mechanism concerning the underlying placental pathology of preeclampsia. Early during placentation, an impairment in trophoblast differentiation and function causes deficient invasion, leading to poorly remodeled uterine spiral arteries and insufficient blood flow to the placenta. The resulting placental hypoxia, oxidative stress, and mechanical damage leads to the release of anti-angiogenic and pro-inflammatory factors into maternal circulation, causing a systemic, symptomatic maternal response.[1–3] Based on this paradigm, numerous factors, including sFLT-1 (soluble fms related receptor tyrosine kinase 1) and sENG (soluble endoglin), have been investigated as potential biomarkers of the disease.[3] While this mechanism is representative of a large number of cases, there is increasing evidence that no one paradigm or predictive biomarker can accurately describe all cases of preeclampsia.[4,5]

This notable heterogeneity has led our group and others to investigate the potential existence of preeclampsia subclasses, driven by different placental pathologies. Using an unsupervised clustering approach that integrates transcriptional and histopathologic profiles of placentas from women across the clinical preeclampsia spectrum, we identified and characterized

3 distinct and clinically relevant disease subtypes. Cluster 1 is largely comprised of healthy placentas, but the preeclampsia subtype it contains (maternal preeclampsia) is primarily driven by maternal factors, with minimal placental dysfunction. Cluster 2 is composed primarily of preeclamptic placentas (canonical preeclampsia), demonstrating transcriptional profiles consistent with the current preeclampsia paradigm of placental hypoxia as the underlying disease pathophysiology, and histological profiles with evidence of maternal vascular malperfusion (MVM). Cluster 3 included mostly preeclamptic samples, demonstrating an intriguing transcriptional and histopathologic profile marked by an increase in immune and pro-inflammatory genes (ie,TNF [tumor necrosis factor]-α and IFN [interferon]-γ), and placental lesions consistent with chronic inflammation and maternal-fetal interface disturbances, such as villitis of unknown etiology (VUE) or massive perivillous fibrin deposition (MPFD).[4–6] As immunological preeclampsia clustered independently from canonical preeclampsia samples, the factors driving the pathology and biomarkers that could be used to diagnose it may also be unique. Preliminarily, we screened the transcriptional profiles of these samples to identify secreted factors possibly mediating immune dysfunction.[5] From this, we identified FGL-2 (fibrinogen-like protein 2) as a potential marker of immunological preeclampsia, from its known roles in immunoregulation and fibrin deposition—2 prominent features of this subclass.

FGL-2 has primarily been investigated as an immunomodulator in the context of transplant tolerance/rejection. In vivo and in vitro, its expression can be upregulated by proinflammatory cytokines, such as TNF-α and IFN-γ, and it elicits an immunosuppressive response on B cells, macrophages, T cells, and regulatory T cells.[7,8] Direct FGL-2 overexpression or treatment with recombinant protein was seen to promote a shift towards type 2 immunity, contributing to allograft tolerance.[9,10] It is essential to cytokine-dependent spontaneous abortion in the CBA X DBA/2 mouse model, and its expression was shown in various trophoblast and leukocyte subsets of the mouse and human decidua and placenta.[8] Additionally, FGL-2 exhibits prothrombinase activity, promoting the formation of fibrin through bypass of the traditional coagulation cascade.[8] Interestingly, both a lack and an excess of FGL-2 were shown to lead to pathological conditions in other systems.[11]

Because both known functions of FGL-2—immunoregulation and fibrin deposition—are intrinsic to healthy or pathological placental development, we hypothesized that FGL-2 is an important mediator in the placental pathophysiology specific to immunological preeclampsia. In this study, we first show, by network analysis, that *FGL2* is part of an immunoregulatory gene module that drives the features of immunological preeclampsia. We then identify clinical features and histopathologic lesions of the placenta that associate with *FGL2* expression. Finally, we determine the broader transcriptional changes enriched in these lesions and explore how *FGL2* could contribute to their development. Finally, we discuss the potential of this secreted factor in the context of previously investigated biomarkers.

Methods

The raw microarray data used in this study are available on the GEO database (GSE75010). Other data or methods may be made available upon request to the authors.

Data Set Assembly and Analysis

Selection and processing of samples from the Research Centre for Women's and Infants' Health BioBank (Mount Sinai Hospital, Toronto, Canada), as well as procedures for microarray and unsupervised clustering analysis of a combined data set were previously described. Clinical information from available samples was collected and analyzed.[4,5] For the present study, after cluster reassignment of cluster 5 samples, the data set was filtered for samples belonging to transcriptional clusters 1, 2, and 3. All analyses were accomplished using RStudio, version 1.1.453 (R version 3.5.0). Network analysis was accomplished using R package *WGCNA*.[12] All gene ontology (GO) analyses were performed using R package g:*Profiler*.[13]

Statistical Analysis

Where multiple groups were compared, normality was tested using the Shapiro-Wilk normality test. As appropriate, Welch 2-sample *t* test or the Kruskal-Wallis rank-sum test, followed by Dunn post hoc test and Benjamini-Hochberg False Discovery Rate adjustment for multiple comparisons, were applied. For correlation analyses, Pearson correlation was used when variables were continuous, and the Spearman rank correlation was used when variables were categorical. Statistical significance was inferred when $P<0.05$.

Detailed methods are available in the Data Supplement.

Results

FGL-2 Is Differentially Expressed Between Transcriptional Clusters

Our original patient cohort included both healthy controls and preeclamptic patients that spanned the clinical spectrum of the disease. We first investigated if placental *FGL2* mRNA expression could discriminate between clinically distinct groups of patients: first between healthy and preeclamptic samples, and second, considering the time of delivery (term versus preterm) as an indicator of preeclampsia disease severity. *FGL2* was unchanged in patient samples with a diagnosis of preeclampsia compared with healthy controls ($P=0.0951$; Figure 1A). Existing studies commonly distinguish between early- and late-onset preeclampsia using a cutoff of 34 weeks of gestation. We found a decrease in *FGL2* expression in samples with early-onset preeclampsia compared with healthy samples ≥34 weeks ($P=0.0276$; Figure 1B). When *FGL2* expression was compared among our previously described transcriptional clusters, a unique pattern of cluster-specific *FGL2* expression emerged ($P=6.794\times10^{-08}$; Figure 1C). Placentas from cluster 2, comprised primarily of canonical preeclampsia patients, showed significantly lower *FGL2* expression ($P=1.1246\times10^{-4}$) than placentas from cluster 1, used as the reference group as it included mostly healthy term control patients.[6] Placentas from cluster 3 had higher *FGL2* expression relative to the same reference group ($P=8.8917\times10^{-04}$). While clusters 2 and 3 are heavily weighted with preeclampsia patient samples, some healthy controls clustered alongside and demonstrated similar placenta histopathology findings, despite no symptomatic evidence of maternal disease. Interestingly, the pattern of cluster-specific *FGL2* expression was unchanged by clinical diagnosis, as *FGL2* expression was similar between healthy and preeclamptic samples within each transcriptional cluster ($P=0.3425$, cluster 1; $P=0.9677$, cluster 2; $P=0.7907$, cluster 3; Figure 1D).

As clinical diagnosis often depends on protein detection, we confirmed this cluster-specific FGL-2 expression at the protein level (Figure 1D). By Western blot, FGL-2 protein was differentially expressed between clusters ($P=0.0362$),
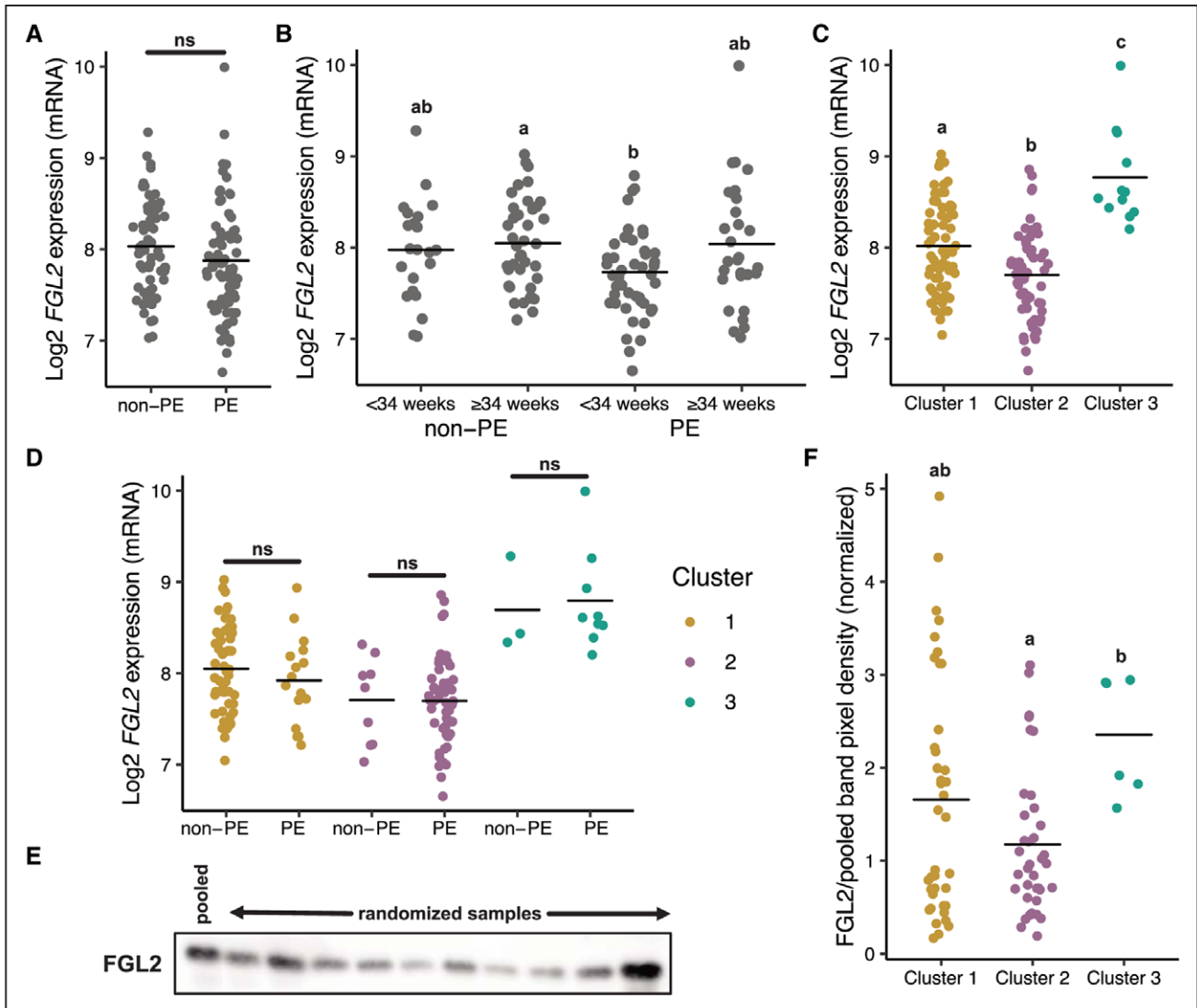
Figure 1. FGL2 (fibrinogen-like protein 2) is differentially expressed among gene expression clusters. A, *FGL2* expression is unchanged between preeclamptic and healthy samples ($P=0.0951$, Welch t test). **B**, *FGL2* expression is lower in preeclamptic samples with a gestational age below 34 wk ($P=0.0435$; Kruskal-Wallis, Dunn post hoc test). Different letters indicate statistical differences between groups: $P=0.4237$ (healthy<34 wk/healthy ≥34 wk), $P=0.0720$ (healthy<34 wk/preeclampsia [PE]<34 wk), $P=0.4980$ (healthy<34 wk/PE≥34 wk), $P=0.0276$ (healthy≥34 wk/PE<34 wk), $P=0.5082$ (healthy≥34 wk/PE≥34 wk), $P=0.0749$ (PE<34 wk/PE≥34 wk). **C**, *FGL2* expression is different among clusters ($P=6.794\times10^{-8}$; Kruskal-Wallis, Dunn post hoc test). Different letters indicate statistical differences between groups: $P=1.1246\times10^{-04}$ (clusters 1/2), $P=8.9817\times10^{-04}$ (clusters 1/3), $P=2.3702\times10^{-07}$ (clusters 2/3). **D**, *FGL2* expression is not different between healthy and preeclamptic samples from the same cluster (ns, nonsignificant, $P=0.3425$, cluster 1, $P=0.9677$, cluster 2, $P=0.7907$, cluster 3; multiple Welch t tests). **E**, Sample Western blot for FGL-2 and its quantification, presented as the ratio of FGL-2 band to pooled sample band pixel density, each normalized to whole lane protein. Pooled sample was run on all blots to eliminate batch effect. **F**, FGL-2 protein expression is different among clusters ($P=0.0362$; Kruskal-Wallis, Dunn post hoc test). Different letters indicate statistical differences between groups: $P=0.0972$ (clusters 1/2), $P=0.0514$ (clusters 1/3), $P=0.0191$ (clusters 2/3). Horizontal bars represent group means.

with a modest decrease in FGL-2 protein expression in cluster 2 ($P=0.0972$) and increase in cluster 3 ($P=0.0514$), although not reaching statistical significance, relative to our cluster 1 reference group.

Placental FGL-2 Is Expressed by Cytotrophoblast, Syncytiotrophoblast, and Intravillous/Intervillous Histiocytes

To gain further insight into the physiological role of *FGL2* in the placenta, we investigated its localization within the placenta by immunohistochemistry. We found FGL-2 expression in the cytotrophoblast and syncytiotrophoblast layers

of the villi (Figure 2A). Furthermore, strong expression was noted in some leukocytes present in fetal vessels within villi and in leukocytes or lymphocytes in the intervillous space (Figure 2B through 2D). CD68 staining confirmed at least a subset of these FGL-2-positive cells to be monocytes/histiocytes (Figure 2B through 2D). No difference in staining localization was observed based on cluster membership.

FGL-2 Is Part of a Gene Module Differentially Enriched Between Preeclampsia Disease Subtypes

Given that the preeclampsia subtypes were derived from transcriptional profiles, the clinical phenotypes likely arise from
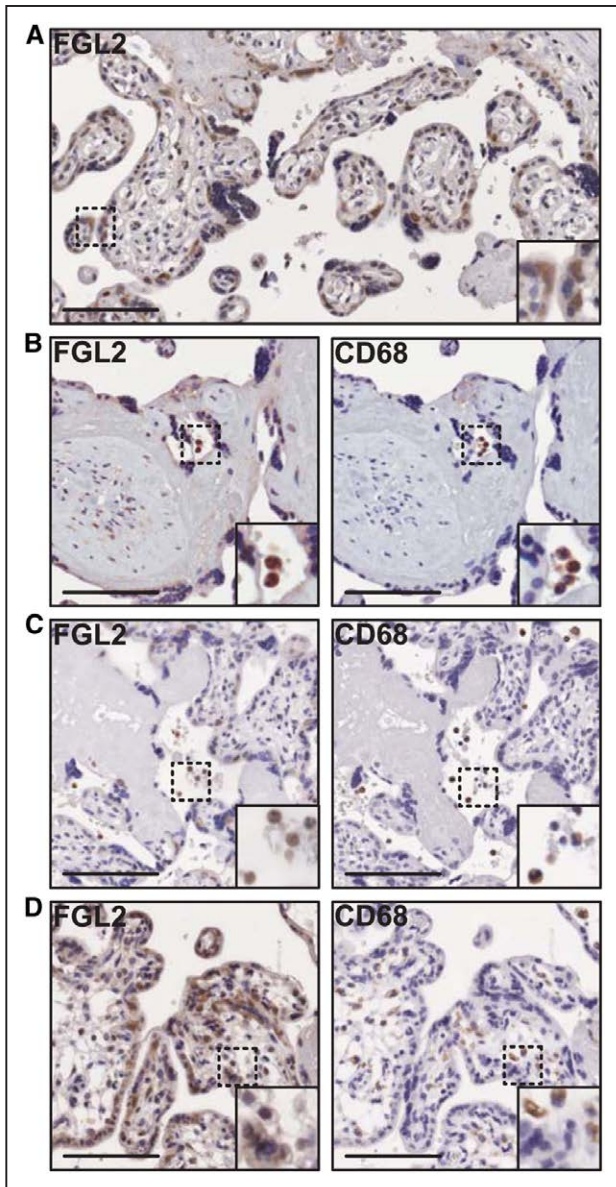
Figure 2. Localization of FGL-2 (fibrinogen-like protein 2) expression within the villous space. Immunohistochemistry staining of villous tissue for FGL-2 and CD68 in serial sections (images are representative of samples from all clusters). A, FGL-2 is expressed by the cytotrophoblast and syncytiotrophoblast layers of the term villi. FGL-2 and CD68 are coexpressed by subsets of (**B**) leukocytes within fetal vessels (**C**) intervillous leukocytes/lymphocytes and (**D**) infiltrated leukocytes/lymphocytes, identifying them as monocytes/macrophages. Scale bars=100μm.

expression of different functional gene sets. To define the contribution of *FGL2* to each disease subtype, we applied Weighted Gene Correlation Network Analysis to our gene expression dataset.[12] This analysis identified 29 modules of coexpressed genes, often indicative of common function. We calculated and compared the enrichment of each module among diagnostic groups: healthy and preeclamptic samples within each gene expression cluster. Broadly, 3 groups of modules with similar enrichment patterns could be observed (Figure 3A). Of interest was a large module (yellow) that contained a panel of several genes that are known preeclampsia markers or have been investigated as such, including *FLT1*,

ENG, INHA/INHBA, FSTL3, PVRL4, NDRG1, SERPINA3, LEP, TREM1.[14–20] GO analysis revealed that yellow module genes are primarily involved in regulating metabolic changes, cell death, and response to hypoxia (Figure 3B). Hub genes of the yellow module, defined as vertices with degree over 30, included genes of this well-established gene panel and several others (Figure 3D).

A few modules were noticeable because of the large difference in their expression between diagnostic groups, suggesting that the expression of these modules is what differentiates one diagnostic group from another. The purple module showed this enrichment pattern, with high expression in immunological preeclampsia samples (within transcriptional cluster 3), compared with healthy samples from the same cluster, and importantly, when compared with canonical preeclampsia samples (within transcriptional cluster 2; Figure 3A). GO analysis revealed overwhelming enrichment for functions related to immunity and leukocyte response (Figure 3C). Interestingly, *FGL2* was found in this purple module, indicating its involvement in a gene set that discriminates between clinically significant diagnostic groups. Network representation of this module showed *FGL2* not to be a hub gene itself but to have 8 direct neighbors: *CD86, CTSS, DOCK10, EVI2B, GPR65, PTPRC, SAMSN1,* and *TLR2* (Figure 3E). Several of these direct neighbors were hub genes of the network, suggesting that while *FGL2* itself may not be a core regulator of the gene set, its expression is closely related to those that are.

FGL-2 Poorly Associates With Pregnancy Outcome Parameters in Preeclampsia Pregnancies

Previously, our group performed a correlative analysis between clinical demographic variables and transcriptional cluster membership.[5] Because of the observed differences in *FGL2* expression among gene expression clusters, we assessed the correlation between *FGL2* expression and the severity of maternal and fetal clinical phenotypes for all cases of diagnosed preeclampsia in our data set. We found poor associations between placental *FGL2* expression and maternal characteristics (maternal age and maternal body mass index, Figure IA and IB in the Data Supplement). *FGL2* was also a poor predictor of the severity of maternal preeclampsia symptoms (HELLP syndrome, maternal mean arterial pressure, and urinary protein, Figure IC through IE). We found a weak but significant positive association between *FGL2* and gestational age at delivery—a proxy of disease severity (Pearson r=0.2504, P=0.0303, Figure IIA). We found no association between *FGL2* and infant birthweight centile (Pearson r=−0.1770, P=0.1519), but interestingly, samples found at both extremes of *FGL2* expression (high and low) demonstrate the lowest birthweight centile. This is indicative of poor fetal outcomes both in samples where placental *FGL2* is abnormally low and where it is abnormally high (Figure IIB). Concurrently, placental *FGL2* expression was not correlated with small for gestational age (SGA)/appropriate for gestational age (AGA) categorization, fetal sex or mode of delivery (Figure IID and IIE).

FGL-2 Expression Is Associated With Chronic Inflammatory Lesions of the Placenta

We next sought to determine the relationship between placental *FGL2* expression and placental histopathology findings, from
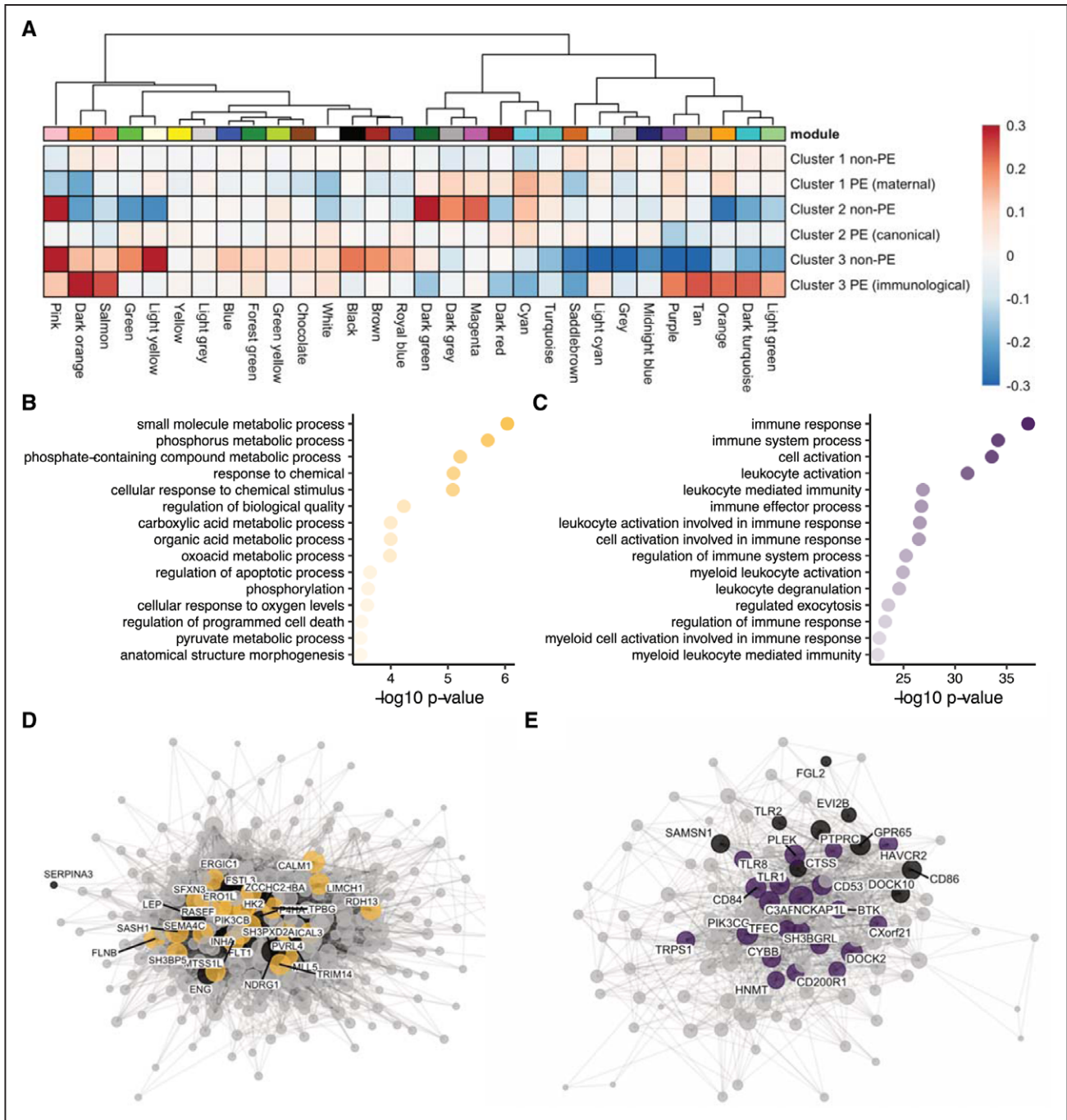
Figure 3. Weighted gene correlation network analysis (WGCNA) reveals discriminating gene modules. A, Gene module enrichment in diagnostic groups (average z-score of module genes for all samples in a diagnostic group). **B**, Yellow module genes relate to metabolic changes and response to hypoxia. **C**, Purple module genes relate to immune response and leukocyte function. **D**, Yellow module (942 genes) comprises a panel of previously investigated preeclampsia (PE) biomarkers, labeled and shown in black. Module hub genes (degree>30) are labeled and shown in yellow. **E**, Purple module (268 genes) comprises *FGL2* and 8 direct neighbors, labeled and shown in black. Module hub genes are labeled and shown in purple.

a blinded, semi-quantitative evaluation by a perinatal pathologist, across all placentas in our cohort.[6] For each lesion scored, we calculated an association score to *FGL2*, using the difference in mean placental *FGL2* expression between placentas with (score≥1) and without (score=0) this lesion. The lesions were subsequently ranked from highest to lowest score, revealing which lesions have the strongest positive and negative associations with placental *FGL2* expression (Figure 4A).

The lesions that most positively associated with placental *FGL2* expression were VUE, delayed villous maturity, chronic deciduitis, maternal inflammation, avascular fibrotic villi, fetal inflammation, and MPFD (Figure 4A and 4B). The majority of these lesions are representative of inflammatory insults within the placenta, suggesting a role for excess *FGL2* in immune dysregulation within the placenta. VUE, chronic deciduitis, and chronic intervillositis
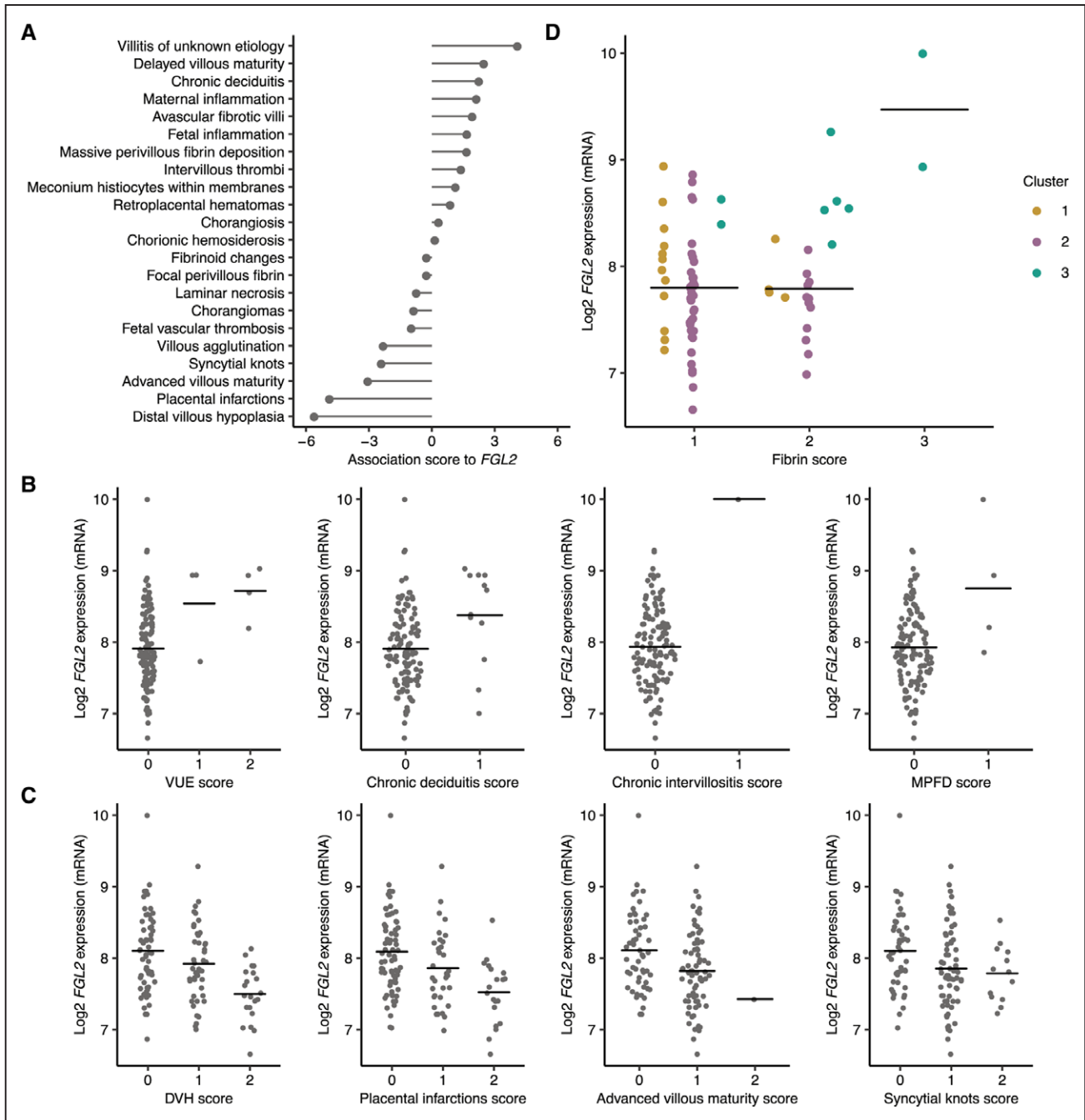
Figure 4. *FGL2* (fibrinogen-like protein 2) expression associates with cluster-specific histopathologic lesions. A, Common placental histopathologic lesions ranked by association score (*t* value: difference in *FGL2* expression, relative to data variation, between affected and nonaffected placentas). **B**, *FGL2* associates positively with chronic inflammation lesions: villitis of unknown etiology (VUE), chronic intervillositis, massive perivillous fibrin deposition (MPFD), and chronic deciduitis. **C**, *FGL2* associates negatively with maternal vascular malperfusion (MVM) lesions: distal villous hypoplasia (DVH), placental infarctions, advanced villous maturity, and syncytial knots. **D**, Samples with the highest fibrin score tend to have higher *FGL2* expression. Semi-quantitative examination of total villi area occupied by perivillous fibrin determined scores: 0=0%, 1≤5%, 2=5%–30%, and 3=30%–50%. Horizontal bars represent group means.

are chronic placental inflammation lesions, while MPFD is categorized as a maternal-fetal interface disturbance lesion that often co-occurs with chronic inflammation lesions.[6,21,22] Previously, these lesions were also shown to be prevalent in the immunological preeclampsia placentas of our cohort.[6] Based on those associations and on *FGL2*'s known role as an immunoregulator and as a prothrombinase, we chose to further investigate those four lesions.

The lesions that most negatively associated with placental *FGL2* expression were distal villous hypoplasia, placental infarctions, advanced villous maturity, and syncytial knots (Figure 4A through 4C). All of these lesions, with the addition of villous agglutination, are representative evidence of MVM, a category of lesions found to be strongly associated with canonical preeclampsia placentas (hypoxia-driven), which have low *FGL2* expression.[6]

Because *FGL2* has known prothrombinase activity and has been shown to promote fibrin deposition in other tissues, and because MPFD only represents extreme cases, we chose to investigate more closely the relationship between *FGL2* expression and perivillous fibrin deposition. A semi-quantitative scoring of the total area of the villi occupied by perivillous fibrin was conducted by a perinatal pathologist. We first observed that the majority of transcriptional cluster 3/immunological preeclampsia samples, which have high *FGL2*, were attributed a fibrin score of 2 or 3 (Figure 4D). In fact, the samples with the highest degree of perivillous fibrin deposition (score of 3) also had the highest *FGL2* expression and belonged to transcriptional cluster 3. Overall, this suggests that placentas with high *FGL2* expression exhibit lesions consistent with chronic inflammatory insults and significant perivillous fibrin deposition.

FGL-2-Associated Transcriptional Profiles of Placental Inflammatory Lesions

We next sought to assess the global transcriptional changes associated with visible changes in the placenta, as evidenced by histopathologic lesions. For all lesions, we conducted differential gene expression analysis between placentas with a histopathology score of 1 or over (lesion present), and those with a score of 0 (lesion absent). We first examined genes found to be differentially expressed in placentas affected by MVM lesions, as negatively associated with placental *FGL2* expression. Among upregulated genes was a panel of genes previously reported as biomarkers of preeclampsia, identified in the yellow module of our network analysis: *FLT1, ENG, INHA/INHBA, FSTL3, PVRL4, NDRG1, SERPINA3, LEP*, and *TREM1* (Figure 5A through 5D). *FGL2* consistently fell within the downregulated group of genes, as did most genes from the purple module and other immune modules. As transcriptional profiles of these 4 lesions were similar, we determined gene ontology reflective of an MVM transcriptional phenotype by using genes that were consistently upregulated across all 4 lesions. Most enriched terms were related to metabolism, proliferation, migration, and negative regulation of immune processes (Figure 5E).

We then examined transcriptional changes in the chronic inflammation lesions that were positively associated with *FGL2* expression: VUE, chronic deciduitis, and MPFD. As only one sample from our cohort was affected by chronic intervillositis, we did not include it in our analysis. We examined upregulated genes, with emphasis on those with strong correlative expression to *FGL2*, as this suggests contribution to common biological processes. We noticed a significant overlap between upregulated genes in VUE, chronic deciduitis, and MPFD, suggestive of a common chronic inflammation transcriptional phenotype as a basis for several related, but distinct, histopathologic lesions (Figure 6A through 6C). The majority of upregulated genes belonged to either the purple or the tan modules—both immune-specific modules identified in our network analysis. Broadly, GO revealed roles in immune response and leukocyte function (Figure 6A through 6C). Among the top upregulated genes in all 3 lesions, an interesting group of IFN-γ-induced genes was prominent: *CXCL9, CXCL10, CXCL11, CXCL13, GBP4, GBP5, RSAD2*, and

IFNG itself. Numerous leukocyte or lymphocyte activation or recruitment factors were present: *ICOS, CD38, CD48, TRAT1, CCL2, CCL5, CCL8,* and *VCAM1*, as well as many genes specific to histiocyte activation or function: *CD74, MSR1, CD86*, and *LYZ*. The genes that were found to be direct neighbors of *FGL2* by network analysis were consistently upregulated in placentas with VUE, chronic deciduitis, and MPFD: *CD86, CTSS, DOCK10, EVI2B, GPR65, PTPRC, SAMSN1,* and *TLR2*. The majority of these genes showed strong correlation to *FGL2*, suggesting its contribution to immune cell-specific, IFN-γ-dependent processes that lead to the development of these lesions. Interestingly, the panel of genes previously reported as biomarkers of preeclampsia, identified in the yellow module of our network analysis (*FLT1, ENG, INHA/INHBA, FSTL3, PVRL4, NDRG1, SERPINA3, LEP,* and *TREM1*), was found to be downregulated in placentas with VUE or chronic deciduitis (Figure 6A and 6B), suggesting they would be ineffective at identifying preeclamptic patients with these types of placental lesions/pathophysiology.

The transcriptional profile of placentas with MPFD was intriguing. Genes from the purple and tan modules, including IFN-γ-induced genes and leukocyte and lymphocyte activation factors, were upregulated. Similarly to the VUE and chronic deciduitis profiles, the majority of these genes correlated well with *FGL2* (Figure 6C), suggesting its contribution to fibrin deposition. Surprisingly, genes of the yellow module, including previously reported biomarkers of preeclampsia, were not downregulated, but upregulated or unchanged, as in placentas with MVM lesions, demonstrating that placentas with MPFD exhibit transcriptional characteristics of both chronic inflammation and MVM lesions. GO for genes upregulated in MPFD reflected this, with terms related to immune response but also to metabolic changes, similar to the terms enriched in yellow module genes (Figure 6C).

Discussion

The clinical presentation and placental pathology observed in cases of preeclampsia exhibit notable heterogeneity. Increasingly, it is becoming apparent that no one paradigm for the pathogenesis of preeclampsia can explain all cases, and no one biomarker can identify them all. Our group has proposed this is due to the existence of disease subtypes with divergent underlying placental pathophysiology, as supported by the identification of distinct patient clusters with high clinical, transcriptional, and histopathologic similarities.[4–6] Investigation of cluster-specific expression patterns of genes and gene networks will help tease apart these distinct physiological processes and importantly, point to potential unique, subtype-specific biomarkers, and therapeutic targets.

In our cohort, placental *FGL2* expression could not discriminate between clinical groups based on diagnosis but was different among previously described transcriptional clusters, particularly high in cluster 3/immunological preeclampsia. Immunological preeclampsia represents a newly described disease subtype with a pathology that likely diverges from the traditional paradigm of preeclampsia. It has a transcriptional profile consistent with profound immune activation, and a histological profile indicative of maternal-fetal interface disturbances and chronic inflammation.[4–6] The known
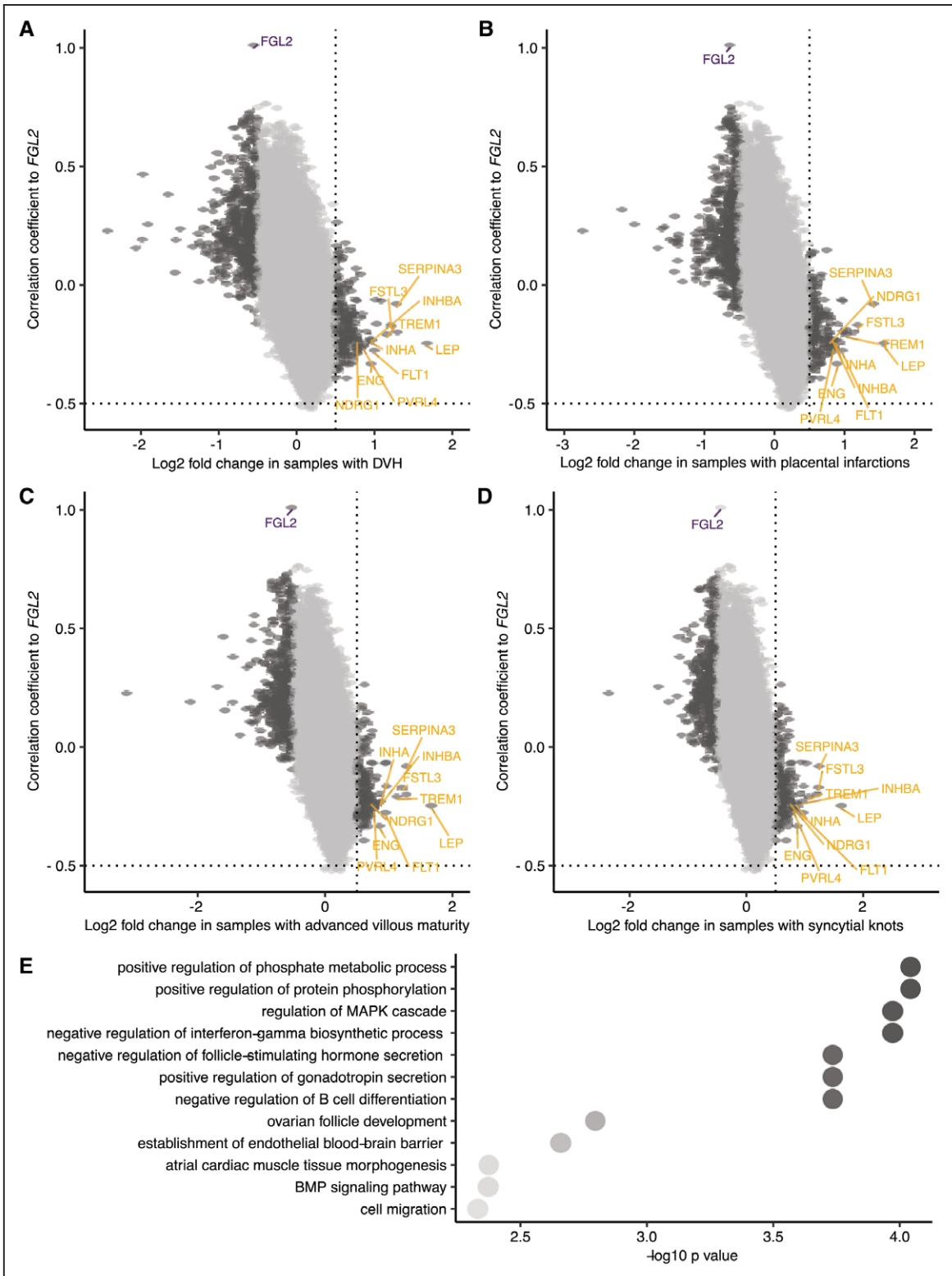
Figure 5. *FGL2* (fibrinogen-like protein 2)-associated transcriptional changes in placentas with maternal vascular malperfusion (MVM) lesions. Differentially expressed genes in placentas with (**A**) distal villous hypoplasia (DVH), (**B**) placental infarctions, (**C**) advanced villous maturity, and (**D**) syncytial knots (correlation coefficient to *FGL2* as a function of log2 fold change in affected placentas). The position of the fold change (0.5) and correlation coefficient (0.5) cutoffs are indicated on panels. Genes of interest are labeled. **E**, Gene ontology (GO) terms for genes commonly upregulated in all 4 MVM lesions indicate a role in metabolic processes and hormonal regulation.

prothrombinase activity of FGL-2 suggests that its excess in affected placentas could contribute to increased fibrin deposition, consistent with findings of MPFD in cluster 3/immunological preeclampsia placentas, while its immuno-modulatory function could contribute to, or be elevated as a result of the activation of immune pathways.[7–9] Considering
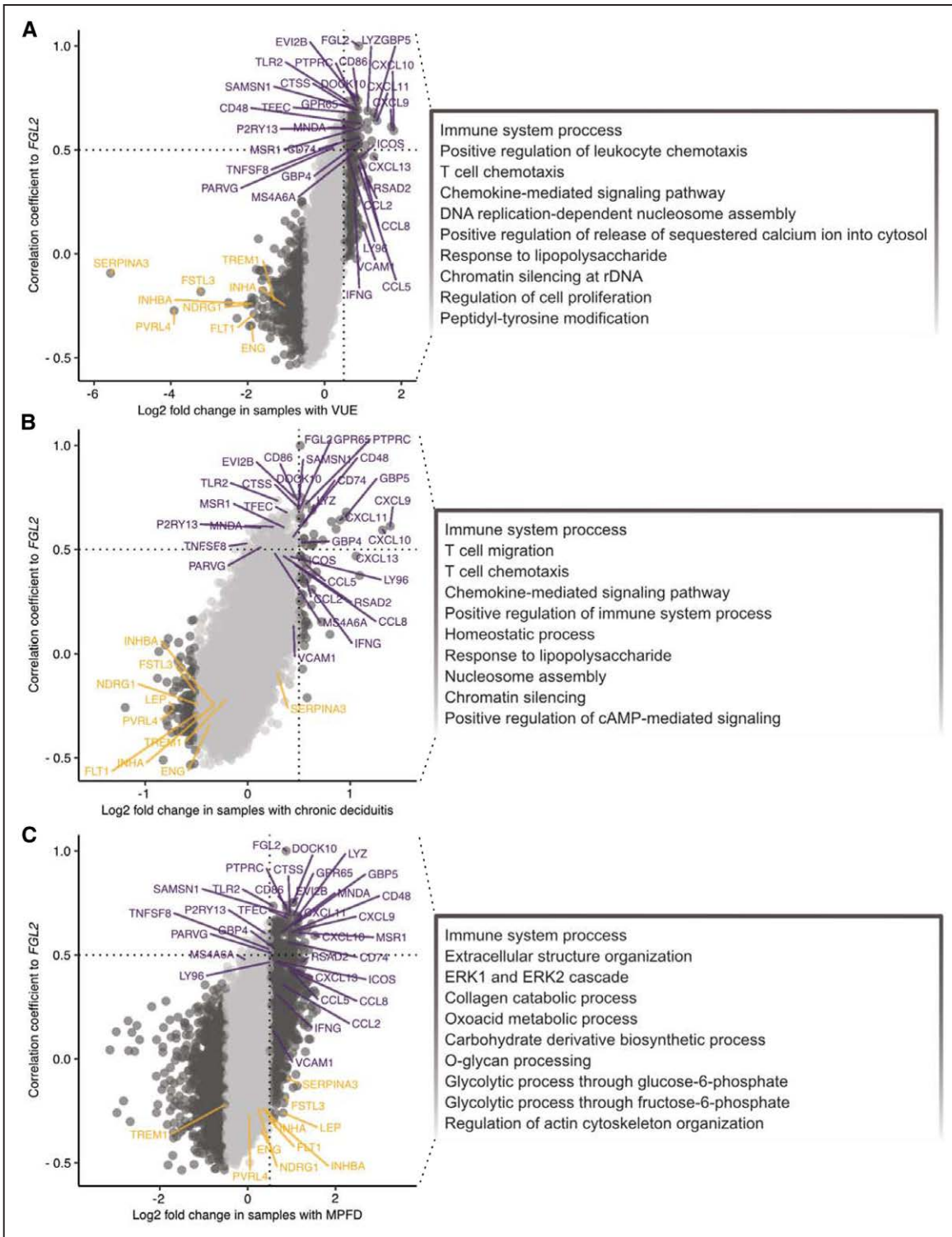
Figure 6. *FGL2*-associated transcriptional changes in placentas with chronic inflammation lesions. Differentially expressed genes in placentas with (**A**) villitis of unknown etiology (VUE), (**B**) chronic deciduitis, and (**C**) MPFD (correlation coefficient to *FGL2* as a function of log2 fold change in affected placentas). Genes of interest are labeled. For each lesion, gene ontology (GO) terms presented are those significantly enriched in upregulated genes, listed from highest to lowest statistical significance.

this, investigation into the role of FGL-2 in pathogenesis in this group of patients is warranted.

To better situate the molecular role of FGL-2 within the preeclampsia subtypes, we applied network analysis, a common method to identify, without bias, functionally relevant groups of genes based on their co-expression across samples.[12] Our approach of module enrichment in diagnostic groups identified gene sets that drive the distinct placental phenotypes observed. The structure of module clustering revealed that immune-focused modules represent the gene sets with

the largest difference in enrichment across diagnostic groups. This analysis indicated that *FGL2* is situated within a highly discriminating module, the purple module. Hub genes from the purple module and the other immune-focused modules are likely drivers of the inflammatory phenotype observed in the preeclamptic samples of transcriptional cluster 3 (immunological preeclampsia). *FGL2* was found on the periphery of the purple module network rather than centrally and highly connected, suggesting that inflammatory pathways driven by these hub genes converge on the observed upregulation of *FGL2*. While this analysis does not constitute evidence of a cause-and-effect relationship between *FGL2* and important immune mediators, this network structure and the identification of *FGL2*'s closest correlation neighbors provide the basis for future, more mechanistic studies of the involvement of *FGL2* in the distinct pathogenesis of immunological preeclampsia.

Anti-angiogenic factors sFLT and sENG have long been suggested as potential biomarkers of preeclampsia and were determined to be highly and consistently upregulated in our canonical preeclampsia samples.[4,5,23] Several other candidate biomarkers have been proposed over the years, and a panel of such genes was found within the yellow module of our network analysis. GO terms enriched in the yellow module mostly related to metabolism and response to hypoxia, which is consistent with the traditional paradigm for the pathology of preeclampsia and with previously conducted gene set enrichment analysis on the clusters of this cohort.[5] The lack of enrichment of this particular module in immunological preeclampsia samples, coupled with strong enrichment of immune-specific ones, further supports the hypothesis that women affected by different preeclampsia subtypes cannot be identified by the same biomarkers.

Severity of pregnancy outcomes varies across clusters-maternal symptoms tend to be more severe in canonical while several fetal outcomes are worst in immunological preeclampsia. The overall trend for a single parameter is driven by the largest group, canonical preeclampsia samples, while the pattern of *FGL2* expression is opposite between subtypes. We think this opposition explains the poor correlations we observed between placental *FGL2* and parameters of pregnancy outcomes.

A recent study from our group has shown overall concordance between transcriptional and histological clustering of samples, suggesting that diagnostic group-specific gene expression patterns, described here, translate to visible changes in placental pathology.[6] MVM lesions are commonly associated with a diagnosis of preeclampsia.[21,24] In our cohort, many of them, including distal villous hypoplasia, placental infarctions, advanced villous maturity, and syncytial knots, were determined to be prevalent in cluster 2/canonical preeclampsia placentas.[5,6] The transcriptional profiles that we obtained for placentas affected by these lesions were consistent with the anti-angiogenic/hypoxic paradigm for the pathology of the disease. Furthermore, a panel of previously investigated biomarkers of preeclampsia (including *FLT1* and *ENG*), observed in the yellow module of our network analysis, was consistently upregulated while *FGL2* was consistently downregulated in MVM-affected placentas.

Chronic inflammatory lesions of the placenta are distinct from acute, infectious lesions in that the cause of the immune insult is often unknown.[21,22] In our assessment, these include VUE, chronic deciduitis, and chronic intervillositis. VUE is relatively common (5%–15%), moderately recurrent (25%–50%), and is associated with intra-uterine growth restriction and intrauterine/neonatal morbidity or mortality.[21,22,25,26] The hypothesis that VUE is the manifestation of maternal immune rejection of the fetoplacental unit is currently favored in the literature: it is characterized by infiltration of maternal T lymphocytes (CD3+, CD4+, CD8+, and regulatory T cells) within the villous stroma, expansion of the fetal Hofbauer cells (intravillous fetal histiocytes), increased maternal monocytes/macrophages within the villous space and a skewed Th1/Th2 cytokine profile.[25–27] Importantly, in affected placentas, expression of MHC class II human leukocyte antigen (HLA) molecules has been observed in the syncytiotrophoblast, which is normally devoid of it.[27,28] This abnormal expression can be induced by excessive IFN-γ.[25,27] Several hub genes from highly discriminating immune modules were upregulated in VUE placentas, where we also observed several IFN-γ-induced genes and several *HLA* genes, which exhibited remarkable correlation to *FGL2*. These findings are suggestive of an IFN-γ-mediated maternal rejection response in placentas affected by VUE and suggest that *FGL2* is involved in this response.

Chronic deciduitis is characterized by lymphocytic infiltrates (largely plasma cells) within the decidua and is associated with preterm labor. It sometimes co-occurs with VUE, and transcriptional and immune cell profiles of placentas with these lesions present a significant overlap.[21,22,29] Our data aligns with these reports, as generally, the *FGL2*-associated transcriptional changes seen in VUE, indicative of a maternal rejection phenotype, were also observed in chronic deciduitis. Strikingly, the transcriptional profiles of both chronic inflammation lesions investigated revealed that in addition to upregulated *FGL2* and maternal rejection-related genes, a panel of previously investigated preeclampsia biomarkers was downregulated. Since this type of lesion is more prevalent in preeclampsia subtype 3/immunological preeclampsia, this suggests that the clinical use of any of these known markers would lead to the omission of patients affected by immunological preeclampsia.

Chronic inflammation lesions are often associated with increased perivillous fibrin deposition, sometimes severe enough to warrant a diagnosis of MPFD. It is been hypothesized that immune insult in MPFD leads to syncytial injury and repair through fibrin deposition. This loss of integrity of the syncytial layer may be at cause in allowing for villous infiltration of maternal T lymphocytes, in placentas affected by chronic inflammation lesions, which would provide a physiopathological link between the 2 types of lesions.[22,26,30] Along with the immunologic basis for the pathogenesis of MPFD, a theory of imbalance between angiogenic and anti-angiogenic factors exists.[31] Our data, interestingly, supports both the immune dysfunction and the angiogenic imbalance hypotheses for this pathology. While *FGL2* and several correlated immune genes were upregulated in the MPFD profile, the panel of previously investigated preeclampsia biomarkers was upregulated or unchanged in affected placentas, contrarily to the VUE/chronic deciduitis profile.

Our finding that previously investigated preeclampsia biomarkers would be poorly predictive of the presence of chronic inflammation lesions, common in immunological pre-eclampsia, highlights the need for subtype-specific biomarkers. *FGL2*-associated transcriptional evidence of a maternal rejection phenotype in these lesions indicates that, as a sub-type-discriminating secreted factor, FGL-2 is a strong candidate and that its mechanism of action should be determined.

The localization of FGL2 expression within the villous space, coupled with its peripheral position in our network analysis, allows us to speculate on this mechanism. In other systems, *FGL2* can be upregulated by pro-inflammatory cytokines TNF-α and IFN-γ, which we have shown are at high levels in immunological preeclampsia and its associated lesions. We propose that this upstream signal upregulates FGL-2 in the cytotrophoblast/syncytiotrophoblast, as a physiological attempt at re-establishing an appropriate immune equilibrium. It is, therefore, likely that, considering its immunosuppressive capacity, high *FGL2* expression dampens the immune response and contributes to pregnancy maintenance, even in unfavorable, inflammatory conditions. The maternal rejection response towards the fetoplacental unit, present in chronic inflammation lesions, could also be at cause in the upregulation of *FGL2*, given the known role of *FGL2* in promoting allograft tolerance.[9,11] The pro-thrombinase activity of FGL-2 has been observed in several studies, where an increase in FGL-2 caused increased fibrin deposition in systems like joints and microvasculature in response to inflammatory stimulation.[32,33] We, therefore, propose that *FGL2* upregulation collaterally promotes fibrin deposition within the villous space.

While this study is limited by the small number of placentas affected by each lesion, in our cohort, these numbers reflect rates of incidence in the general population, in most cases. Transcriptional profiles and correlations should be confirmed in other cohorts, and individual genes and gene regulatory networks identified here will require further mechanistic validation. This study identifies considerable differences in transcriptional and histopathologic patterns between clusters of our cohort, highlighting the need to consider disease subtypes in future studies of preeclampsia biomarkers. Immunological preeclampsia, particularly, is unlikely to be predicted by markers, such as sFLT or sENG. Genes from highly discriminating, immune-specific modules offer potential and FGL-2, as a secreted immunomodulator and prothrombinase, is a promising candidate.

Perspectives

Our previous discovery and characterization of gene expression clusters and preeclampsia subtypes laid the foundation for the investigation of cluster-specific expression patterns of genes and gene networks. *FGL2* was selected for its prothrombinase and immunomodulator functions and its intriguing pattern of expression in our cohort. A complete evaluation of the association between its expression and transcriptional, clinical, and histopathologic features of patients and placentas is presented here, substantially adding to our understanding of the specific pathology underlying immunological preeclampsia.

Disclosures

None.

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Novelty and Significance

What Is New?

- *FGL2* is part of a gene module that discriminates between preeclampsia subtypes.
- *FGL2* is upregulated in immunological preeclampsia, and its expression is associated with numerous transcriptional, clinical, and histopathologic features specific to this subtype.
- Placentas affected by chronic inflammation lesions, prevalent in immunological preeclampsia, show a downregulation of common preeclampsia markers, but an upregulation of *FGL2* and *FGL2*-correlated immune regulation genes.

What Is Relevant?

- The discovery of genes involved in subtype-specific disease features enhances our understanding of the diverging pathophysiologies leading to different preeclampsia subtypes (canonical versus immunological), and may ultimately lead to the identification of subtype-specific biomarkers and treatments.

Summary

We have characterized the role of FGL2, a secreted prothrombinase and immunomodulator, in the placental pathology specific to immunological preeclampsia, furthering our understanding of this unique and noncanonical disease subtype.